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Interaction of the Hepatitis B Spliced Protein with Cathepsin B Promotes Hepatoma Cell Migration and Invasion

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Hepatitis B spliced protein (HBSP) is involved in the pathogenicity and/or persistence of hepatitis B virus (HBV). Chronic HBV infection is one of the most important risk factors for the development of hepatocellular carcinoma (HCC). However, whether or not HBSP contributes to the progression of HBV-associated HCC remains unknown. This study reports that overexpression of HBSP in human hepatoma cells increased cell invasion and motility. Conversely, small interfering RNA (siRNA)-mediated knockdown of HBSP expression inhibited migration and invasion. By glutathione *S*-transferase (GST) pulldown, coimmunoprecipitation, and a mammalian two-hybrid assay, HBSP was found to directly interact with cathepsin B (CTSB). Similar to HBSP knockdown, knocking down CTSB also reduced cell migration and invasion. Furthermore, the HBSP-overexpressing hepatoma cells were shown to have increased expression and activity of matrix metalloproteinase-9 (MMP-9) and urokinase-type plasminogen activator (uPA), and overexpression of HBSP significantly enhanced tumor-induced vascularization of endothelial cells. In contrast, knockdown of either HBSP or CTSB by siRNA resulted in inhibition of the two proteolytic enzymes and of the *in vitro* angiogenesis. Expression of HBSP in the hepatoma cells appeared to activate the mitogen-activated protein kinase (MAPK) and Akt signaling pathway, as evidenced by increases in phosphorylation of p38, Jun N-terminal protein kinase (JNK), extracellular signal-regulated kinase (ERK), and Akt. Taken together, these findings imply that interaction of HBSP with CTSB may promote hepatoma cell motility and invasion and highlight new molecular mechanisms for HBSP-induced HCC progression that involve the secretion and activation of proteolytic enzymes, increased tumor-induced angiogenesis, and activation of the MAPK/Akt signaling, thereby leading to the aggressiveness of hepatoma cells.

Chronic hepatitis B virus (HBV) infection has been proven to be one of the most important risk factors for the development of hepatocellular carcinoma (HCC) (3, 9). However, the pathogenesis of cancer in HBV infection is still not fully understood, and it appears that multiple factors and cellular signaling pathways are involved in hepatocarcinogenesis (28). Integration of HBV genome into host DNA can lead to alterations in cellular gene function or generate chromosomal instability (1, 10, 43). Expression of some oncogenic HBV proteins such as HBx and truncated Pre-S2/S has been shown to have a direct effect on malignant transformation of the liver (42) and to promote metastasis of the malignant cells, and there is thus a very high mortality rate for HCC patients (18). Another HBV protein, encoded by a singly spliced 2.2-kb HBV DNA and referred to as the hepatitis B spliced protein (HBSP), is found to be expressed in liver biopsy tissues from four out of five chronic patients but not from two hepatitis C virus-infected patient samples and one normal liver sample (39). Our prior study also showed that a 2.2-kb splice variant was present in all tumor and peritumor samples from 12 HCC patients studied (20). Exogenous expression of HBSP in transfected Huh-7 cells can induce cell apoptosis (40). However, the cytopathic effect of the HBSP was unclear, and its role in progression, invasion, and metastasis of HBV-related HCC has not yet been elucidated.

We previously showed that cathepsin B (CTSB) was one of the major intracellular interacting partners of HBSP, and it was identified using a yeast two-hybrid screening assay (8). This led us to investigate whether the HBSP could interact with CTSB in the context of hepatoma cells to affect cell migration, invasion, and metastasis, all of which can be modulated by CTSB acting directly and indirectly on extracellular matrix (ECM) component remodeling and degradation (26, 47). CTSB is a lysosomal cysteine protease that plays an important role in physiological protein turnover and processing (19, 41). In nonmalignant cells, CTSB is mainly stored in the lysosome, whereas in malignant cells, CTSB redistributes into exocytic vesicles at the cell periphery, leading to its secretion and association with binding partners on the tumor cell surface (22, 33). CTSB can cleave and activate a wide variety of substrates in proteolytic pathways that increase neoplastic progression. It has been shown that activation of urokinase-type plasminogen activator (uPA) and matrix metalloproteinases (MMPs) by CTSB not only enhances ECM degradation and cancer cell motility and invasion (14) but also induces *in vitro* angiogenesis (2). In addition to interacting with other proteases, CTSB may regulate the activity of kinase signaling networks, cell surface receptors, and signaling molecules such as chemokines, cytokines and growth factors (25). However, such interactions are usually bidirectional because kinases can also regulate many proteases through phosphorylation while proteases can control the actions of a multitude of kinases (21).

High expression levels of CTSB have been linked to aggressiveness and poor prognosis in several cancers, such as colorectal and ovarian carcinomas (15, 29, 34, 37, 38). Compared to the amount

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of information on the role of CTSB in colon and ovarian cancers, very little information about the precise function of CTSB in HCC and its interactions with other signaling molecules and pathways regulating the progression of HCC is available. In this study, we attempted to investigate the interaction of HBSP with CTSB both *in vitro* and *in vivo*, examine the function of this interaction by manipulating their expression in various hepatoma cell lines, and explore potential molecular mechanisms underlying the changes in hepatoma cell migratory behaviors and metastatic potential. We found that HBSP interacting with CTSB enhances the migration and invasion of hepatoma cells and promotes *in vitro* angiogenesis via activation of MMP-9 and uPA and the pathways involving mitogen-activated protein kinase (MAPK) and Akt signaling.

MATERIALS AND METHODS

Vector construction. A 2.2-kb spliced, defective HBV DNA was previously isolated from a patient with chronic HBV infection (8). The CTSB and cathepsin D (CTSD) genes were reverse transcribed from the total RNA isolated from Huh-7 cells and cloned into pCMVTNT at the XhoI and SalI sites of the vector (Promega, Madison, WI). The paired forward and reverse primers were 5′-CCGCTCGAGGCCACCTGGCAGCTCTG GGCCTCCCT-3′ and 5′-ACGCGTCGACTTAGATCTTTTCCCAGT-3′ (for CTSB) and 5′-CCGCTCGAGCAGCCCTCCAGCCTTCTGC-3′ and 5′-ACGCGTCGACTTAGAGGCGGGCAGCCTCG-3′ (for CTSD). pACT-CTSB, used for the mammalian two-hybrid assay, was generated by cloning the CTSB gene into the SalI and NotI sites of pACT (Promega, Madison, WI). p3×FLAG-CMV-10-HBSP, used for establishing a stable HBSP-expressing cell line, was constructed by the insertion of an HBSP open reading frame (ORF) into p3×FLAG-CMV-10 (Sigma, St. Louis, MO).

Cell lines and transfection. The human hepatoma cell lines Huh-7, HepG2, and Hep3B and human dermal microvascular endothelial cell line HMEC-1 (obtained from the American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and maintained in a humidified atmosphere containing 5% CO_2 at 37°C. Transfection was performed using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions.

Generation of stable HBSP-expressing hepatoma cell lines. To establish stably transfected cell lines expressing HBSP N-terminally fused to 3×FLAG, the Huh-7, HepG2, and Hep3B hepatoma cells were transfected with the respective vectors and selected in the presence of 800 μg/ml Geneticin for 4 weeks. The Geneticin-resistant clones were individually expanded into cell lines and screened for the extent of HBSP protein expression by Western blotting.

Western blot analysis. Cell lysates were prepared using radioimmunoprecipitation assay (RIPA) protein lysis buffer (Pierce Company, Rockford, IL), and the protein extracts were quantified and then subjected to electrophoresis on a 4 to 15% SDS-PAGE gel. The proteins were transferred to polyvinylidene difluoride (PVDF) membranes and blocked in Tris-buffered saline (TBS) containing 5% bovine serum albumin (BSA). After the membranes were incubated sequentially with primary and secondary antibodies, the immunoreactive protein bands were visualized using CDP-Star reagents (Roche, Mannheim, Germany) and analyzed densitometrically by a ChemiImager (Alpha Innotech Corporation, San Leandro, CA). The specific antibodies used in this study included anti-FLAG (1:2,000; Sigma), anti-GAL4 (1:1,000; Clontech, Palo Alto, CA), anti-CTSB, anti-MMP9 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), anti-uPA, anti-p38, anti-pp38, anti-Jun N-terminal protein kinase (anti-JNK), anti-pJNK, anti-extracellular signal-regulated kinase (anti-ERK), anti-pERK, anti-Akt, and anti-pAkt (1:1,000; Cell Signaling Technology, Beverly, MA).

***In vitro* cell invasion assay.** The invasive capabilities of cells were determined using 24-well BD BioCoat Matrigel invasion chambers (BD Biosciences, San Jose, CA). Cells were serum starved overnight, and an aliquot of 5×10^4 cells in 500 μl DMEM was added to the upper chamber of transwell while 750 μl DMEM containing 10% FBS and 10 μg/ml fibronectin (BD Sciences, San Diego, CA) were added to the lower chamber. After 36 h of incubation, Matrigel and cells remaining in the upper chamber were manually removed by cotton swabs, and the inserts were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet. Cells in at least six randomly selected microscopic fields were counted and photographed. All experiments were performed in duplicate and repeated five times.

Wound healing migration assay. Cells were grown to 90% confluence on 6-well plates, and a scratch through the cell monolayer was introduced by using a pipette tip. Baseline (time zero) images were captured and cell migration was assessed at 48 h by counting the number of cells that had migrated across the scratch, and this number was normalized to the scratch area. Data were expressed as percentages of the control value. Cell migration data were obtained from five independent wound healing experiments.

GST pulldown assay. Construction, expression, and purification of glutathione *S*-transferase (GST)-fused HBSP or HBx were described previously (8, 12). An *in vitro* T7-coupled reticulocyte lysate system (Promega) was used to generate ^{35}S-labeled CTSB protein by the addition of 2 μg of pCMVTNT-CTSB with 50 μCi of [^{35}S]methionine (Amersham Pharmacia Biotech, Arlington, IL). ^{35}S-labeled CTSB was added to the GST-HBSP recombinant-protein-immobilized beads and incubated overnight at 4°C. Beads were washed three times with 1% Triton X-100 in phosphate-buffered saline (PBS), resuspended in SDS sample buffer, and subjected to 12% SDS-PAGE. ^{35}S-CTSB was detected by autoradiography. Both GST-fused HBx protein and ^{35}S-labeled CTSD generated by pCMVTNT-CTSD were used as irrelevant negative controls.

Co-IP assay. For the *in vivo* coimmunoprecipitation (Co-IP) experiments, transiently transfected Huh-7 cells were lysed and the soluble proteins were precleared with 100 μl of a 50% slurry of protein A agarose (Invitrogen). The clear lysates were then mixed with 2 μg of goat polyclonal anti-CTSB IgG (Santa Cruz Biotechnology) and 100 μl of a 50% slurry of protein A agarose. The immunoprecipitated complexes were analyzed by 10% SDS-PAGE and immunoblotting using anti-GAL4 DNA-binding domain (GAL4 BD) monoclonal antibody (Clontech) and anti-CTSB antibody (Santa Cruz Biotechnology).

Mammalian two-hybrid assay. A mammalian two-hybrid assay was performed using the CheckMate mammalian two-hybrid system (Promega) according to the manufacturer's protocol. Briefly, pACT-CTSB and each of the pBIND-HBSP, pBIND-HBSP$_{1-47}$, and pBIND-HBSP$_{48-111}$ vectors were cotransfected with the pG5*luc* reporter plasmid into Huh-7 cells by using Lipofectamine 2000 (Invitrogen). Paired empty plasmids pBIND and pACT were used as negative controls. Cells were harvested 48 h posttransfection, and renilla-normalized firefly luciferase activities were measured using the Dual-Luciferase reporter assay system (Promega).

RNA interference assay. The small interfering RNA (siRNA) specifically targeting HBSP (HBSP-siRNA) or the human CTSB (CTSB-siRNA) was designed at the Whitehead Institute webserver (http://sirna.wi.mit.edu/) and chemically synthesized by Shanghai GenePharma Co. (Zhangjiang Hi-Tech Park, Shanghai, China). A nontargeting siRNA (NC-siRNA) was used as a negative control. Transfections were performed using the Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions.

Real-time PCR analysis. Total RNA was extracted using the TRIzol reagent (Invitrogen) and converted to cDNA by using the ExScript reverse transcription-PCR (RT-PCR) kit (TaKaRa, Japan). Quantitative real-time PCR was performed in the ABI StepOne real-time PCR system (Applied Biosystems, Foster City, CA) in the presence of SYBR Premix *Ex Taq* (TaKaRa). The GAPDH (glyceraldehyde-3-phosphate dehydrogenase)

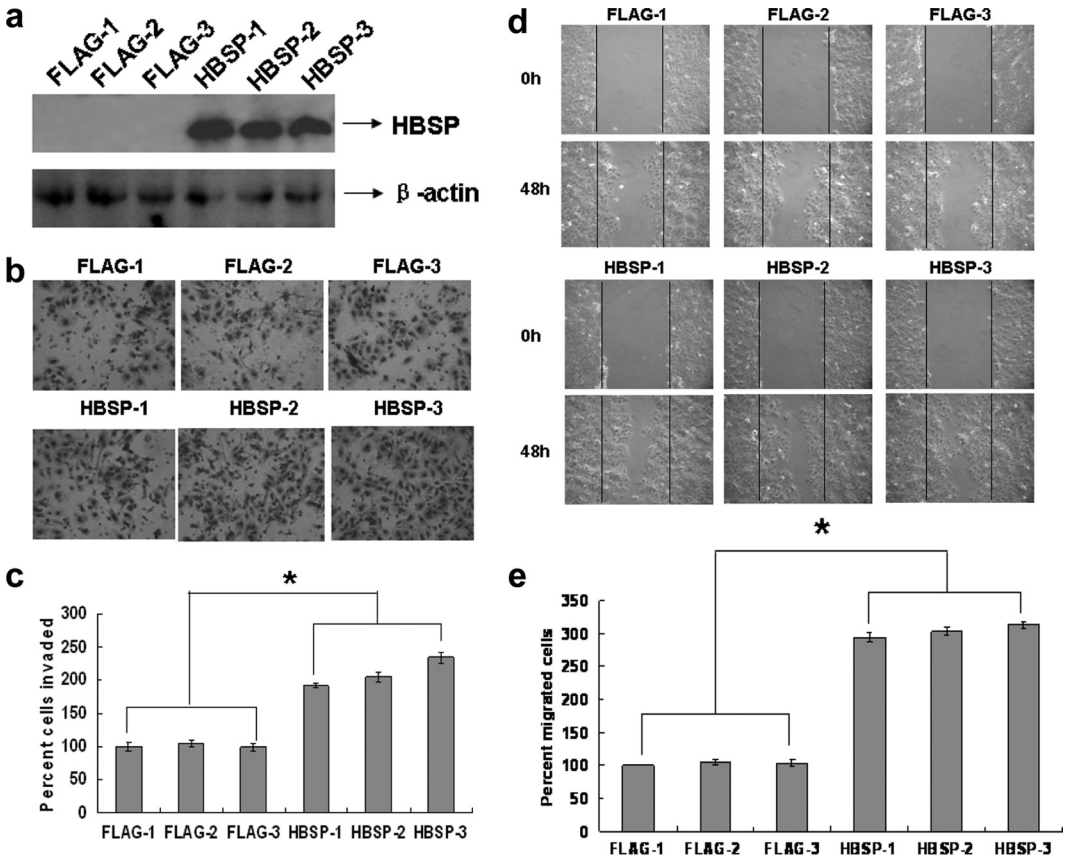

FIG 1 HBSP enhances invasion and migration of hepatoma cells. (a) The three individual clones stably transfected with p3×FLAG-CMV-10-HBSP (HBSP-1, HBSP-2, and HBSP-3) or those stably transfected with empty control p3×FLAG-CMV-10 (FLAG-1, FLAG-2, and FLAG-3) were randomly selected and examined for the presence of HBSP protein by Western blotting. (b) The Matrigel invasion assay was performed with the three HBSP-positive clones and the three negative controls. Thirty-six hours after incubation, the invaded cells were stained with crystal violet, photographed, and counted. (c) The number of invaded cells in each well was counted from six microscopic fields and expressed as a percentage of the control value. Values are means ± SDs ($n = 5$). *, $P < 0.01$ (versus controls). (d) Relative motility as visualized by the ability of the cells to close a wound made by creating a scratch through a lawn of confluent cells. (e) Cell migration was assessed at 48 h by counting the number of cells that had migrated across the scratch, normalized to the scratch area and expressed as a percentage of FLAG-1 controls. Values are means ± SDs ($n = 5$). *, $P < 0.05$ (versus controls).

gene was used as a reference gene, and relative mRNA levels were determined using the $2^{-\Delta\Delta CT}$ method. The paired forward and reverse primers were 5′-ACCTCGAACTTTGACAGCGACA-3′ and 5′-GATGCCATTCACGTCGTCCTTA-3′ (for MMP-9), 5′-CTGTGAGATCACTGGCTTTG-3′ and 5′-TTGGAGGGAACAGACGAG-3′ (for uPA), and 5′-TGCACCACCAACTGCTTAGC-3′ and 5′-AGCTCAGGGATGACCTTGCC-3′ (for GAPDH).

Gelatin zymography. Activities of MMP-9 in the culture medium of cells were analyzed using gelatin zymography. Cells were serum starved for 24 h, after which the cell-conditioned medium was collected and equal amounts of proteins were then separated under nonreducing condition on 10% zymogram gels containing 0.1% gelatin (Invitrogen). After electrophoresis, the gel was incubated in the zymogram-renaturing buffer for 30 min at room temperature, followed by an overnight incubation at 37°C in the zymogram-developing buffer. The gel was then stained with Coomassie blue R-250 (Sigma) for 30 min and destained with the destaining solution for 1 h. Clear bands appear on the Coomassie blue R-250-stained blue background in the areas of gelatinolytic activity.

***In vitro* angiogenesis assay.** An *in vitro* angiogenesis assay was implemented as described previously, with some modifications (24). Briefly, conditioned medium was collected from the transfected cells grown in serum-free medium for 16 h and used to culture HMEC-1 cells, which were seeded in 96-well plates coated with 50 μl Matrigel and incubated for 16 h. The formation of the microtubule networks was examined and photographed. The angiogenic effect was measured by counting the lengths of capillary tubes in five different fields and expressed as a percentage of the control value.

Statistical analysis. Statistical analysis was performed using SPSS software (SPSS 17.0; SPSS Inc., Chicago, IL). Data are expressed as the means ± standard deviations (SDs). Statistical significance ($P < 0.05$) of differences between groups was determined by Student's *t* test.

RESULTS

HBSP enhances the invasion and migration of hepatoma cells. To explore the functional significance of the HBSP protein in hepatoma cells, we first examined the effects of its expression in Huh-7 cells on cell invasive and migratory ability. Three individual clones from the Huh-7 cells stably transfected with p3×FLAG-CMV-10-HBSP or an empty control (p3×FLAG-CMV-10) were randomly selected for the following studies. As shown in Fig. 1a, p3×FLAG-CMV-10-HBSP-transfected clones HBSP-1, HBSP-2, and HBSP-3 expressed a strong band corresponding to the HBSP protein, whereas this band was not detectable in the empty-vector-transfected clones FLAG-1, FLAG-2, and FLAG-3. The effect on invasive potential was examined using a modified Boyden chamber invasion assay. The number of cells that invaded through

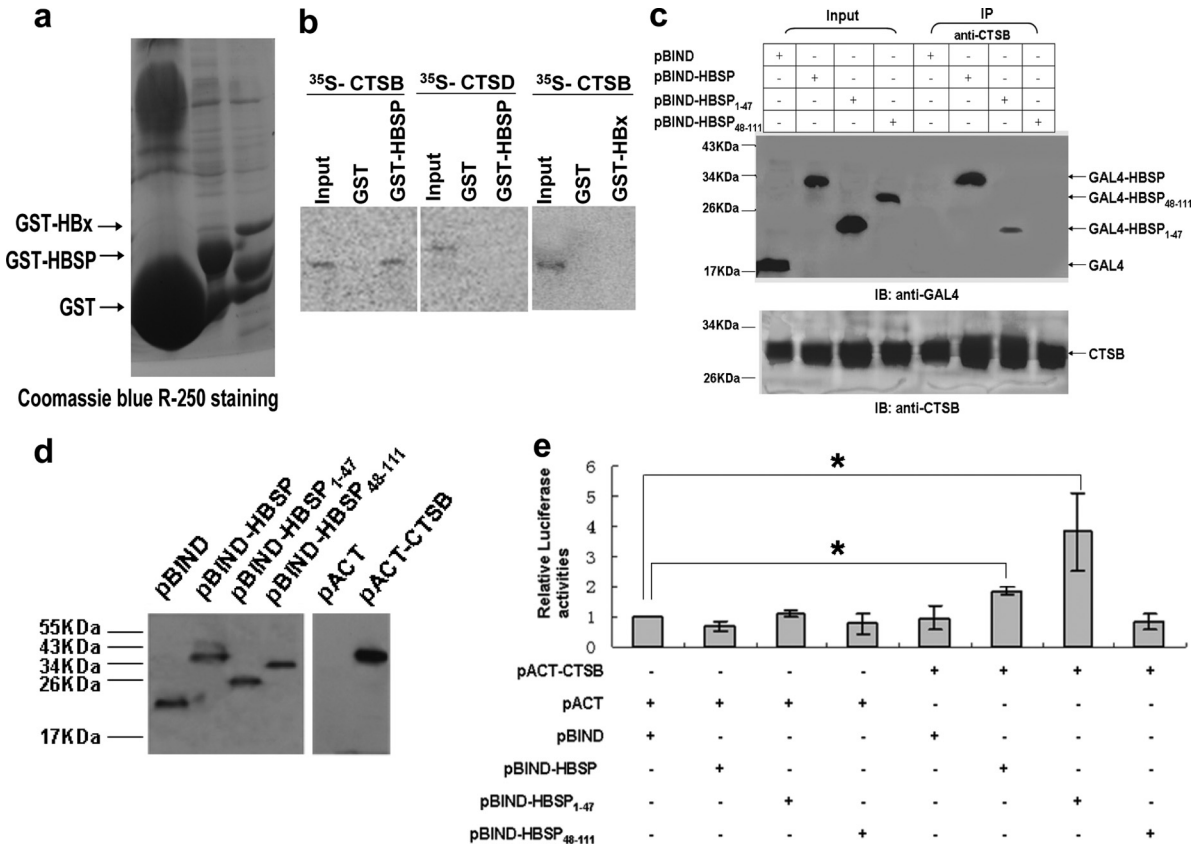

FIG 2 *In vitro* and *in vivo* interactions between HBSP and CTSB. (a) Coomassie blue-stained SDS-PAGE gel showing that GST, GST-HBSP, and GST-HBx recombinant proteins were expressed in *E. coli* and bound to glutathione Sepharose beads. (b) Representative autoradiogram of *in vitro*-translated ^{35}S-CTSB captured by GST-HBSP fusion proteins from a GST pulldown assay. Several independent experiments yielded consistent results. The input lane was loaded with 1/10 the amount of ^{35}S-labeled proteins used in the binding reactions. GST-HBx fusion protein and ^{35}S-CTSD were used as irrelevant negative controls, demonstrating that HBSP and CTSB bound specifically under the conditions employed. (c) Coimmunoprecipitation of HBSP and endogenous CTSB after Huh-7 cells were transfected with pBIND-HBSP, pBIND-HBSP$_{1-47}$, or pBIND-HBSP$_{48-111}$ and immunoprecipitates (IP) were immunoblotted (IB) for GAL4 (upper panel) or CTSB (lower panel). (d) Western blot analysis confirmed expression of full-length and truncated HBSP fused with GAL4 DNA-binding domain (left panel) and CTSB fused with VP16 activation domain (right panel) in Huh-7 hepatoma cells. (e) *In vivo* association of HBSP with CTSB. A mammalian two-hybrid assay was used to assess the interaction between HBSP and CTSB in Huh-7 cells cotransfected with same amount of pBIND-HBSP or pBIND containing truncated-HBSP variants, pACT-CTSB and pG5*luc*, and measured for luciferase activity. Empty vectors pBIND and pACT together with pG5*luc*-cotransfected cells are the negative controls. Values are means ± SDs ($n = 5$). *, $P < 0.01$ (versus controls).

a layer of Matrigel was analyzed at 36 h after the cells were plated on Matrigel-coated transwell inserts. Figure 1b and c show that compared with empty-vector-transfected controls, HBSP-expressing cells significantly increased their invasive potential. Likewise, HBSP-expressing cells migrated through the wound scratch more rapidly than the control cells (Fig. 1d and e). Owing to the fact that all three clones demonstrated very similar results, HBSP-3 cells were randomly chosen for all subsequent experiments. The FLAG-1 cells were used as a negative control.

To further confirm that HBSP expression promotes invasion and migration of hepatoma cells, another two HCC cell lines (HepG2 and Hep3B) were used to assess the effects of HBSP expression on migratory behaviors. Similarly, when HBSP was expressed in HepG2 and Hep3B cells, cell invasion and migration were enhanced significantly in the HBSP-expressing HepG2 and Hep3B cells compared with those of vector control cells (data not shown). This result indicates that the effect of HBSP on cell migration and invasion is not confined to a single hepatoma cell model.

HBSP interacts with CTSB both *in vitro* and *in vivo*. We have previously shown the evidence of interaction between HBSP and CTSB in a yeast two-hybrid system (8). To test the association between these two proteins *in vitro*, we carried out a GST pulldown assay. As shown in Fig. 2, GST and the fusion proteins GST-HBSP and GST-HBx were expressed in *Escherichia coli* and immobilized on glutathione Sepharose beads (Fig. 2a). ^{35}S-labeled CTSB, but not the irrelevant ^{35}S-labeled CTSD protein, was retained when reacted with bead-bound GST-HBSP fusion protein, while ^{35}S-labeled CTSB was not retained with bead-bound GST-HBx control protein (Fig. 2b).

To confirm and extend the result of the GST pulldown assay, we performed an *in vivo* Co-IP study with the Huh-7 cells transiently transfected with a pBIND vector that expressed full-length HBSP, HBSP$_{1-47}$ (lacking the 64-residue C-terminal domain [amino acids 48 to 111]), or HBSP$_{48-111}$ (lacking the 47-residue N-terminal domain [amino acids 1 to 47]). As shown in Fig. 2c, full-length HBSP and HBSP$_{1-47}$, but not HBSP$_{48-111}$, were able to interact with endogenous CTSB, as evidenced by efficient coimmunoprecipitation with the antibody to CTSB (upper panel, lanes 6 and 7 from the left), indicating that the N-terminal 47 amino

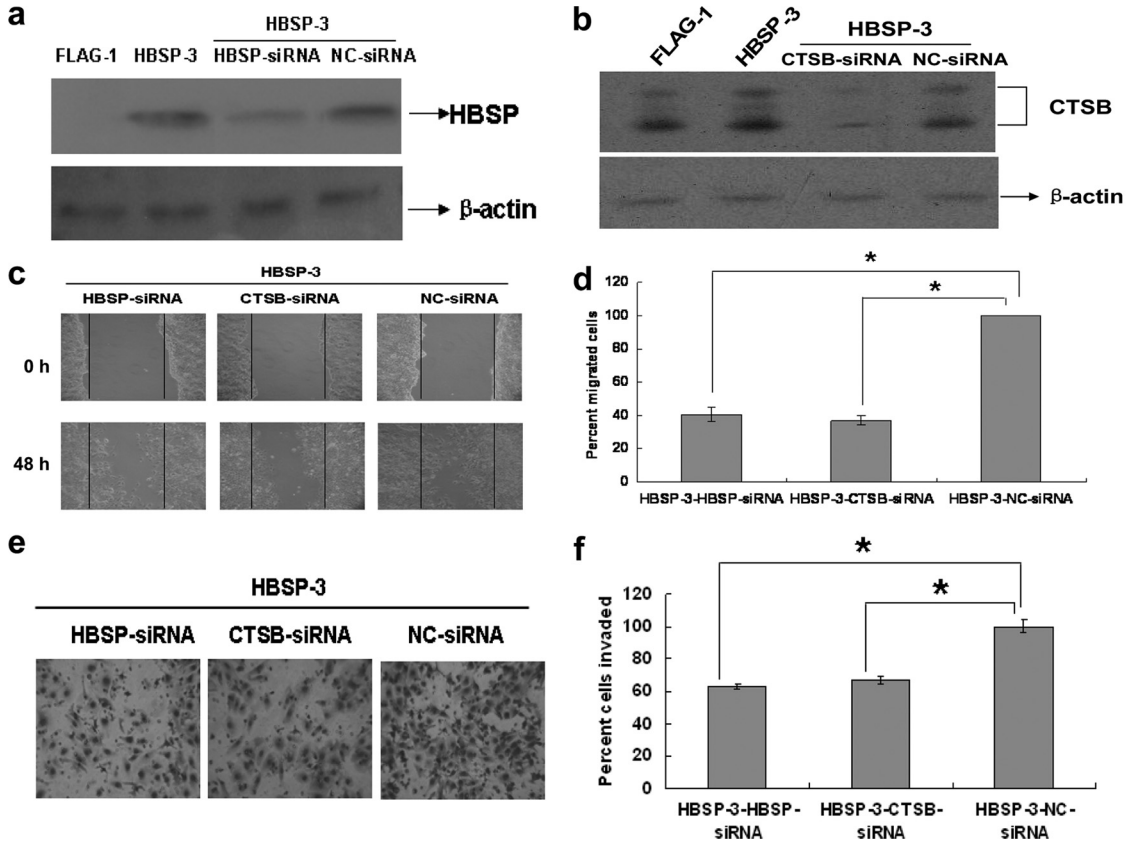

FIG 3 Downregulation of HBSP or CTSB expression inhibits hepatoma cell invasion and migration. siRNA-mediated knockdown of HBSP (a) or CTSB (b) expression in HBSP-3 cells. (c) Relative motility as determined by the ability of the HBSP-3 cells, when HBSP or CTSB was knocked down, to close a wound made by creating a scratch through a lawn of confluent cells. (d) Cell migration was assessed by counting the number of cells that had migrated across the scratch. The number of migrated cells was normalized to the scratch area and expressed as a percentage of the nontargeting siRNA control value. Values are means ± SDs ($n = 5$). *, $P < 0.01$ (versus controls). (e) The Matrigel invasion assay was performed with the HBSP-siRNA, CTSB-siRNA, and NC-siRNA cells. Thirty-six hours after incubation, the invaded cells were stained with crystal violet, photographed, and counted. (f) The number of invaded cells in each well was counted from six microscopic fields and expressed as a percentage of the control. Values are means ± SDs ($n = 5$). *, $P < 0.01$ (versus controls).

acid residues of HBSP were indispensable for its interaction with CTSB.

In order to confirm the coimmunoprecipitation data and to further validate the domains of HBSP necessary and sufficient for interactions with CTSB, we carried out HBSP-CTSB interaction studies using the mammalian two-hybrid system. Immunoblot analysis revealed that each protein was efficiently expressed in the Huh-7 hepatoma cells when fused to the GAL4 BD or VP16 activation domain (AD) (Fig. 2d). Significantly increased luciferase activity was observed in cells cotransfected with pBIND-HBSP or pBIND-HBSP$_{1-47}$ and pACT-CTSB (Fig. 2e). In contrast, there was no discernible difference between the luciferase activities of the pBIND-HBSP$_{48-111}$ group and negative control (Fig. 2e). These results indicate that the N-terminal 47 amino acid residues of HBSP participate in the interaction with CTSB, which confirms the results of the coimmunoprecipitation study.

Interaction of HBSP and CTSB contributes to the invasion and migration of hepatoma cells. Given that expression of HBSP in Huh-7 cells promotes motility and that we have demonstrated the existence of its interaction with CTSB, we examined the effects of knockdown of either HBSP or CTSB on cell migration and invasion. Small interfering RNAs (siRNAs) targeting HBSP or CTSB expression were separately introduced into HBSP-3 cells. An siRNA containing a sequence that does not target any known mammalian gene was used as a control. The expression levels of HBSP and CTSB were analyzed by Western blotting, which showed marked reductions in HBSP and CTSB protein levels (Fig. 3a and b) in HBSP-3 cells. Wound healing and transwell invasion assays were again used to assess the effects of HBSP and CTSB expression on cell motility. Compared with control siRNA-transfected cells (negative control), HBSP-3 cells with HBSP-siRNA or CTSB-siRNA showed significantly impaired cell migration in the wound healing assay (Fig. 3c and d) and cell invasion through the Matrigel (Fig. 3e and f). These results provide further evidence that HBSP and CTSB may function in a way to promote the migration and invasion of the hepatoma cells.

Interaction of HBSP and CTSB increases expression of MMP-9 and uPA. Recent evidence has revealed that proteases, once regarded as little more than cellular garbage disposals or ECM degraders, play more and more important roles in regulating multiple processes during malignant progression, including angiogenesis, invasion, and metastasis, etc. CTSB has been implicated in a proteolytic cascade through interactions with MMPs and the serine protease uPA (14). The fact that high-level expression of proteases has been found to be correlated with invasive growth in many human malignancies suggests that this does not

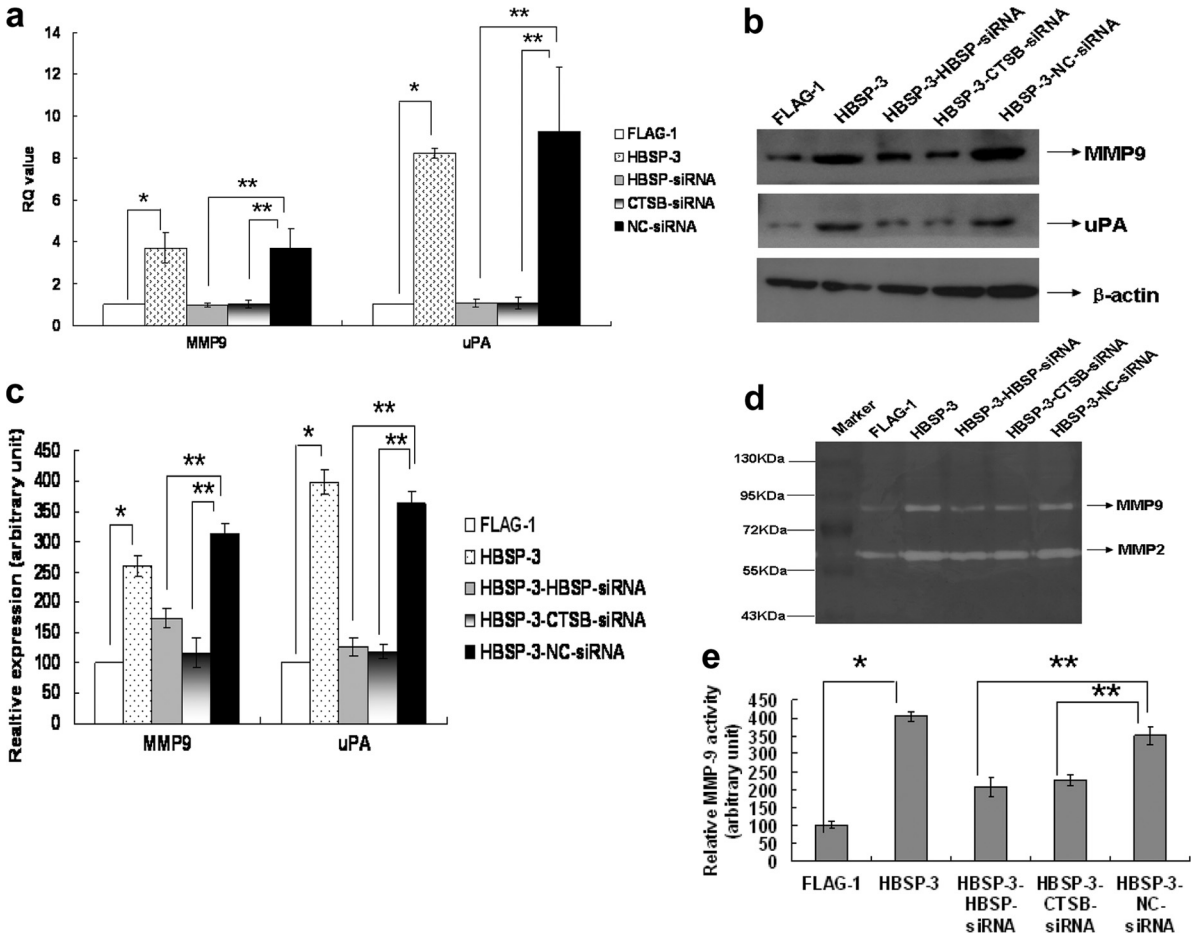

FIG 4 Interaction of HBSP and CTSB increases expression of MMP9 and uPA. (a) The relative mRNA levels (RQ) of MMP-9 and uPA in FLAG-1, HBSP-3, HBSP-3–HBSP-siRNA, HBSP-3–CTSB–siRNA and HBSP-3–NC-siRNA cells were measured by quantitative RT-PCR. Values are means ± SDs ($n = 5$). *, $P < 0.01$ (versus FLAG-1 negative control); **, $P < 0.01$ (versus NC-siRNA control). (b and c) Western blot analysis of MMP9 and uPA expression in the five cell lines. The protein expression levels in the HBSP-3, HBSP-3–HBSP-siRNA, and HBSP-3–CTSB-siRNA cells relative to that in the FLAG-1 cells was calculated densitometrically after normalization to the level of β-actin. Values are means ± SDs ($n = 5$). *, $P < 0.01$ (versus FLAG-1 negative control); **, $P < 0.01$ (versus HBSP-3–NC-siRNA control). (d and e) Gelatin zymography to determine the activities of MMP-9 in the cells was performed as described in Materials and Methods. Bands or signals detected by zymography were quantified using densitometric analysis. Values in panel e are means ± SDs ($n = 5$). *, $P < 0.01$ (versus FLAG-1 negative control); **, $P < 0.01$ (versus HBSP-3–NC-siRNA control).

occur by chance. To understand the potential mechanisms underlying HBSP-induced increased invasion and migration of hepatoma cells, the relative expression levels of MMP9 and uPA in the HBSP-3 cells were quantified by quantitative reverse transcription-PCR (qRT-PCR). The mRNA levels of MMP9 and uPA in the HBSP-3 cells were 3.69-fold and 8.23-fold higher, respectively, than those of control cells (Fig. 4a). Conversely, when HBSP or CTSB was knocked down in the HBSP-3 cells by siRNA, the expression of MMP-9 or uPA was significantly reduced. Consistent with the changes in mRNA level, the level of MMP-9 or uPA protein also changed accordingly with the manipulations of HBSP and CTSB expression (Fig. 4b and c). Thus, both HBSP and CTSB may regulate expression of MMP-9 and uPA at the mRNA and protein levels.

Next, gelatin zymography was utilized to evaluate the enzymatic activity of MMP-9 (Fig. 4d and e). Both active forms of MMP-2 (62 kDa) and MMP-9 (82 kDa) were detectable in culture-conditioned media from all the cell lines used, including the controls. Specifically, enzymatic activity of MMP-9 in HBSP-3 cells was enhanced compared to that in FLAG-1 control cells. On the other hand, siRNA knockdown of HBSP or CTSB led to a moderate decrease in the MMP activity present in these cells.

Interaction of HBSP and CTSB promotes *in vitro* angiogenesis. Angiogenesis is an important aspect of tumor progression and metastasis. CTSB, MMP, and uPA are well known to be overexpressed and implicated in angiogenesis during tumor progression (2, 23). To assess the effects of HBSP-CTSB interactions on tumor-induced vessel formation, HMEC-1 cells were cultured in the conditioned medium collected from transfected or control cells. Figure 5a and b show that the endothelial cells formed well-defined capillary-like networks after 16 h of growing under the conditioned medium from HBSP-3 cells. In contrast, the conditioned media harvested from cells transfected with HBSP-siRNA or CTSB-siRNA decreased endothelial capillary formation. These results suggest that both HBSP and CTSB are likely to play prominent roles in tumor-induced angiogenesis *in vitro*.

Interaction of HBSP and CTSB interferes with intracellular signaling events of MAPK and PI3K pathways. To identify pos-

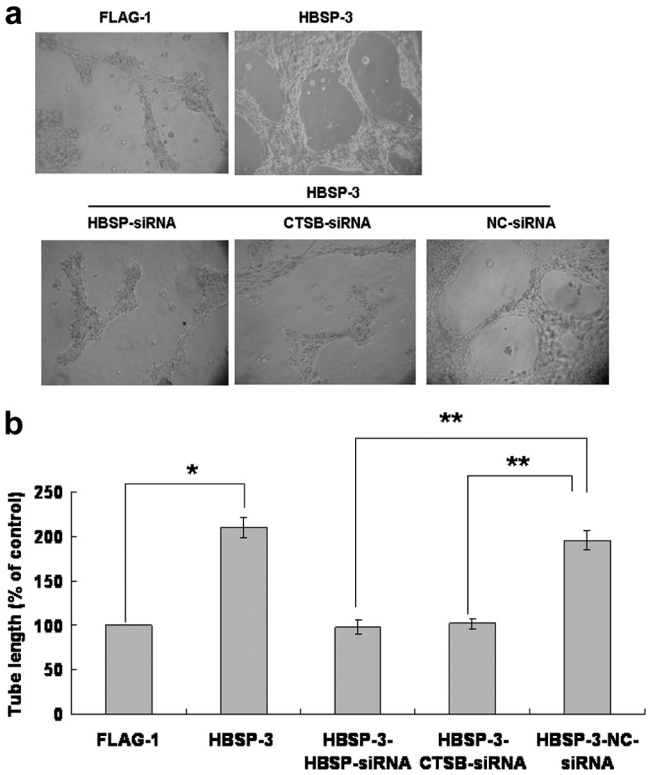

FIG 5 Interaction of HBSP and CTSB promotes *in vitro* angiogenesis. (a) Tumor-induced *in vitro* angiogenesis. The capillary tube formation in HMEC-1 cells was observed and photographed under light microscope, and tube length was calculated in each microscope field. (b) Changes in neovasculature were quantitated by evaluating the length of capillary-like tubes. Values are means ± SDs ($n = 5$). *, $P < 0.01$ (versus FLAG-1 negative control); **, $P < 0.01$ (versus HBSP-3–NC-siRNA control).

sible signaling molecules in HBSP- and CTSB-mediated regulation of hepatoma cell migration and invasion, we examined the expression of molecules involved in MAPK and phosphatidylinositol 3-kinase (PI3K) pathways that are frequently implicated in cell migration, protease induction, regulation of apoptosis, and angiogenesis (7, 32). Western blot analysis was performed to assess the total and phosphorylated levels of p38, JNK, ERK, and Akt in the HBSP-3 cells and siRNA-transfected cells by using specific antibodies. As shown in Fig. 6a and b, significant increases in the levels of phosphorylation of p38, JNK, ERK, and Akt were found in the HBSP-3 cells, and such increases can be completely or partially reversed by transfection of HBSP-siRNA and CTSB-siRNA into the cells. It is important to note that the total protein levels remained essentially the same among all the groups, implying that this was a result of a significant increase in phosphorylated proteins themselves. These findings indicate that HBSP and CTSB activate MAPK and PI3K pathways that may contribute to enhanced invasion in hepatoma cells.

DISCUSSION

The vast majority of all cancer deaths are due to cancer metastasis rather than the influence of the primary tumors. HCC patients have a high incidence of tumor recurrence and metastasis. As a result, the 5-year survival rate for HCC patients is poor. The main cause of death in HCC patients is intrahepatic metastasis, but the underlying mechanisms remain largely unknown. A better understanding of the molecular events governing the pathogenesis of cancer metastasis in HCC is desperately needed in order to improve clinical management and prognosis of this deadly disease. Recently, the cysteine protease cathepsin B has been associated with metastasis and poor prognosis in multiple cancer types, including cervical, colorectal, and ovarian carcinomas and high-grade glioma (15, 23, 26, 29, 34, 37, 38, 45). Using a proteomic signature model, Lee et al. have identified cathepsin B as one of six biomarkers allowing discrimination of HCC from nonmalignant and normal liver tissues with high accuracy (19). However, what role this protein serves in HCC development and progression has not been explored by this group or other investigators.

In our previous study, we searched for novel HBSP binding partners by yeast two-hybrid assay using HBSP as bait to screen a human liver cDNA library and succeeded in identifying CTSB as 1 of 20 HBSP-binding candidates (8). In the present study, we performed comprehensive studies to confirm the interaction of HBSP with CTSB. Using the GST pulldown assay, we found that HBSP, under the experimental conditions, was able to bind to CTSB *in vitro*. To assess the importance of this interaction *in vivo*, HBSP was transfected in Huh-7 cells, and coimmunoprecipitation analysis showed that HBSP interacted *in vivo* with endogenous CTSB and that this interaction was mediated by the N-terminal 47 amino acid residues of HBSP, which was further confirmed in a mammalian two-hybrid system.

CTSB has been shown to be causally involved in migration and invasion of human osteosarcoma and glioblastoma cells (16, 27). In this study, siRNA-mediated knockdown of CTSB in HBSP-expressing HBSP-3 cells reduced cell migration and invasion compared to this action in controls, indicating that enhanced cell motility by HBSP may be due to its interaction with cathepsin B that may play a causative role in determining the invasive potential of hepatoma cells.

Tumor cell invasiveness involves cell attachment, proteolysis of ECM, and migration of cells through the disrupted matrix. Degradation of the ECM by tumor cells is believed to be among the most important of the initial steps for invasion. It has been demonstrated that CTSB can activate uPA and MMPs to increase ECM degradation and tumor cell invasion in an ovarian cancer cell model (14, 30). Here we found that expression of HBSP in hepatoma cells was accompanied by increased expression of uPA and MMP-9 at both the mRNA and protein levels. Conversely, knockdown of either HBSP or CTSB significantly reduced their mRNA and protein expression levels. This suggests that a major component of the effect of HBSP and CTSB on uPA and MMP-9 expression is at the transcriptional level. Proteolytic remodeling of extracellular matrix has been found to be a key event in vessel sprouting during angiogenesis (5, 13) and is mediated by the interaction of proteases and uPA receptor (uPAR) (17, 23). It is well established that angiogenesis plays a pivotal role in the development, progression, and metastasis of various tumors. In particular, HCC is characteristic of hypervascularity and strong tendency to invade vasculature. Thus, targeting angiogenesis in HCC has become an important strategy to treat HCC and interfere with its progression although the development of effective antiangiogenic therapeutics for HCC patients remains a daunting challenge (6). Vascular endothelial growth factor (VEGF) has been identified as the predominant regulator of HCC angiogenesis in association with the tumor grade, vascular invasion, and prognosis (31, 35,

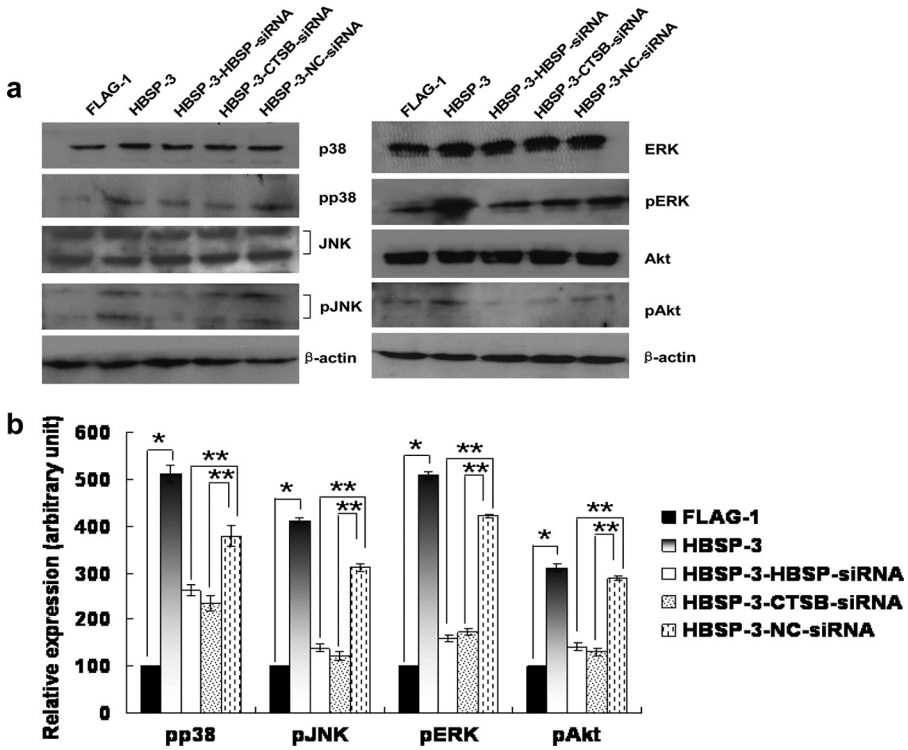

FIG 6 Interaction of HBSP and CTSB affects intracellular signaling events. (a) Western blot analysis showing that phosphorylation of p38, JNK, ERK, and Akt in the HBSP-3 cells was increased compared to that in the FLAG-1 control and that knockdown of either HBSP or CTSB reduced the level of phosphorylation. (b) Relative phosphoprotein levels were normalized to the corresponding total protein levels quantified by densitometry. The ratio for control FLAG-1 cells is designated as 100. Values are means ± SDs ($n = 5$).

46); however, the complex process of angiogenesis involves many other proangiogenic and antiangiogenenic factors acting on both tumor cells and endothelial cells. Notably, we found that knockdown of HBSP or CTSB expression effectively inhibited tumor-induced angiogenesis in the cocultured endothelial cells. The detailed mechanisms by which HBSP-CTSB regulates the protease expression and *in vitro* angiogenesis remain to be defined, but it is noteworthy that disruption of HBSP-CTSB protein interaction may be a potential therapeutic approach for clinical development in the treatment of advanced HBV-related HCC.

HCC development is a multistep process in which multiple pathways are deregulated. Among them, the PI3K/Akt and Ras/MAPK pathways are two of the most commonly and aberrantly activated (4, 44). Kinases and phosphatases have long been understood to interact in regulatory networks such as those proposed for proteases and their endogenous inhibitors. Interestingly, CTSB has been found to be involved in activation of signaling cascades of MAPK and PI3 kinase in the context of tumor cell proliferation and metastasis (11, 36). Our findings revealed that the phosphorylation of p38, JNK, ERK, and Akt in the HBSP-3 cells was increased in the HBSP-expressing cells and can be abolished by the introduction of siRNA against HBSP or CTSB. The results of this study indicate that HBSP-CTSB interaction might activate both MAPK and Akt signaling pathways to increase cell motility.

In summary, we provide experimental evidence unveiling a previously unknown interaction of HBSP with CTSB and suggesting that HBSP can have an effect similar to that of CTSB on mediating cell migration and invasion, which may contribute to the aggressive phenotype of hepatoma cells in a physiological setting.

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